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C. elegans CPB-3 interacts with DAZ-1 and functions in multiple steps of germline development

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Abstract

Cytoplasmic polyadenylation element-binding proteins (CPEBs) are well-conserved RNA-binding proteins, which regulate mRNA translation mainly through control of poly(A) elongation. Here, we show that CPB-3, one of the four CPEB homologs in *C. elegans*, positively regulates multiple aspects of oocyte production. CPB-3 protein was highly expressed in early meiotic regions of the hermaphrodite gonad. Worms deficient in *cpb-3* were apparently impaired in germ cell proliferation and differentiation including sperm/oocyte switching and progression of female meiosis. We also show that *cpb-3* is likely to promote the meiotic entry in parallel with *gld-3*, a component of one of the redundant but essential genetic pathways for the entry to and progression through meiosis. Taken together, CPEB appears to have a conserved role in the early phase of meiosis and in the sperm/oocyte specification, in addition to its reported function during meiotic progression.

Keywords: *C. elegans*; CPEB; Meiosis; Oogenesis

Introduction

Eukaryotic species produce specialized cells called gametes, which convey genetic information to the next generation. In animals, primordial germ cells segregate from somatic cells in an early phase of embryonic development. They proliferate and then differentiate into mature gametes, namely sperm and oocytes. Proliferation and differentiation of germ cells are often controlled through translation of mRNA, which in many cases involves specific regulatory elements lying in the 3′ or 5′ untranslated region (UTR) (Wickens et al., 2000).

A number of germline-specific regulators for translation have been characterized in the nematode *C. elegans*, especially concerning the mitosis/meiosis decision and the sperm/oocyte switch (Crittenden et al., 2003). In the mitosis/meiosis decision, the GLD-1/NOS-3 and the GLD-2/GLD-3 pathways are proposed to promote the entry to meiosis in parallel (Eckmann et al., 2004; Hansen et al., 2004). GLD-1 is a STAR/GSG/quaking-type RNA-binding protein that functions as a translational repressor (Jan et al., 1999; Lee and Schedl, 2001), and NOS-3 is one of the three *C. elegans* homologs of *Drosophila* Nanos (Kraemer et al., 1999). GLD-2 is the catalytic subunit of an atypical poly(A) polymerase (PAP), which acts together with GLD-3, a homolog of Bicaudal-C (Wang et al., 2002). It has been proposed that FBF proteins, which belong to the PUF RNA-binding protein family, repress translation of both *gld-1* mRNA and an isoform of *gld-3* mRNA, when germ cells are to continue mitotic divisions (Crittenden et al., 2002; Eckmann et al., 2004). In the sperm/oocyte switch of hermaphrodite germlines, FBF together with NOS-3 represses *gld-1* and *fem-3* mRNAs and promotes oogenesis (Crittenden et al., 2002; Kraemer et al., 1999; Zhang et al., 1997). GLD-3 is critical to continue spermatogenesis, in which it antagonizes FBF (Eckmann et al., 2002). In addition to these proteins involved in the mitosis/meiosis decision and the sperm/oocyte switch, RNA-binding proteins associated with the germ granules called P-granules, such as PGL and GLH proteins, are important for germline development (Gruidl et al., 1996; Kawasaki et al., 2004; Kuznicki et al., 2000).

We have reported previously that a germline-specific RNA-binding protein DAZ-1 is essential for oogenesis but not for

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spermatogenesis in *C. elegans*. The *daz-1* gene is a member of the *Deleted in azoospermia* (*DAZ*) family, which encode putative translational activators essential for gametogenesis in invertebrates and vertebrates. Oogenic germ cells in the *daz-1* mutant exhibit some irregularities early in meiosis and eventually arrest at the pachytene stage of meiosis (Karashima et al., 2000; Maruyama et al., 2005). DAZ-1 shows its highest expression in the mitotic and the early meiotic regions of the adult hermaphrodite gonad, suggesting that DAZ-1 is likely to function in early steps of female gametogenesis.

Our screen for DAZ-1-interacting proteins has identified another RNA-binding protein CPB-3. In this paper, we describe the role of *C. elegans* CPB-3 in germline development. CPB-3 is one of the four *C. elegans* homologs of the cytoplasmic polyadenylation element-binding protein (CPEB), the others being CPB-1, CPB-2, and FOG-1 (Luitjens et al., 2000). CPEBs, which are characterized by two RNA recognition motifs (RRM) and one zinc-finger domain composed of C4 and C2H2 motifs, were initially identified as regulators of translation in *Xenopus*. In *Xenopus* oocytes, translation of maternal mRNA is regulated by cytoplasmic polyadenylation: dormant mRNAs contain short poly(A) tails, and elongated poly(A) tails stimulate translation. CPEB is a key factor to control polyadenylation and translation. While CPEB is always bound to mRNAs, its polyadenylation-inducing activity depends upon phosphorylation by the kinase Aurora A. Unphosphorylated CPEB acts as a translational repressor by associating directly with Maskin, which in turn binds eIF4E. This Maskin-eIF4E interaction prevents binding of eIF4G to eIF4E and thus inhibits translation initiation. When CPEB is phosphorylated, CPEB recruits CPSF (Cleavage and polyadenylation specificity factor), which then recruits PAP and causes elongation of poly(A). Elongated poly(A) tails disrupt the Maskin-eIF4E association and stimulate translation to induce oocyte maturation (Mendez and Richter, 2001). A *Drosophila* CPEB homolog Orb is required for localization and translation of *gurken* mRNA in dorsoventral axis formation and also for translation of *oskar* mRNA in anteroposterior axis formation (Castagnetti and Ephrussi, 2003; Chang et al., 1999, 2001).

In *C. elegans*, CPB-1 protein is expressed and functional during spermatogenesis. FOG-1 protein is also detectable in spermatogenic cells and is involved in spermatogenesis as well as in proliferation of germ cells (Luitjens et al., 2000; Thompson et al., 2005). *cpb-2* mRNA is known to be enriched in spermatogenic cells, whereas *cpb-3* mRNA is enriched in oogenic cells (Luitjens et al., 2000). Among the four CPEBs, CPB-3 is structurally most similar to the CPEB members implicated in oogenesis in *Drosophila*, clam, zebrafish, *Xenopus*, and mouse (Mendez and Richter, 2001).

Here, we show that CPB-3 has multiple roles during germline development. CPB-3 protein is highly expressed in the early meiotic region of a hermaphrodite germline and seems to be involved in at least two decisions of germ cell fates: the sperm/oocyte switch and the mitosis/meiosis decision. *cpb-3* acts upstream of *fem-3* in the sperm/oocyte switch and in parallel with *gld-3* in the mitosis/meiosis decision. Our findings suggest that CPEB plays an important role in the early phases of germ cell sex determination and meiosis.

Materials and methods

Two-hybrid analysis

The yeast two-hybrid screen was performed using yeast strain PJ69-4A, which contained three reporter genes *ADE2*, *HIS3* and *lacZ* (James et al., 1996). Briefly, yeast cells carrying the Gal4-BD-DAZ-1 plasmid (pAS2C-DAZ-1) were transformed with a random-primed *C. elegans* cDNA library constructed with the vector lambda ACT (Kraemer et al., 1999). We divided 1,300,000 transformants into 75 batches, cultured them briefly, and selected adenine-prototrophs (Ade^+) from them. Four Ade^+ colonies were picked up from each batch, and they were examined for histidine prototrophy. Plasmids were recovered from 282 His^+ transformants. Sequences of the cloned cDNAs were determined by a standard method.

Nematode strains and culture

C. elegans was maintained and manipulated genetically as described (Brenner, 1974). Strains used were wild-type *C. elegans* var. Bristol, strain N2; (LGI), *cpb-3(bt17*, *tm1746)*, *glp-4(bn2)*, *hT2[qIs48]*; (LGII) *daz-1(tj3)*, *fbf-1 (ok91)*, *gld-3(ok308)*, *mIn1[dpy-10(e128) mIs14]*; (LGIV) *fem-3(e2006)*. Strains were maintained and analyzed at 20°C, except that *cpb-3(bt17*, *tm1746)* was analyzed at 20°C and 25°C, and that temperature-sensitive mutants *glp-4(bn2)* and *fem-3(e2006)* were maintained at 15°C and analyzed at 25°C.

Isolation of a cpb-3 deletion mutant using the TMP/UV method

A worm library mutagenized with a combination of trimethylpsoralen and UV irradiation (Yandell et al., 1994) was screened for a deletion in the *cpb-3* locus according to the protocol distributed by Moulder and Barstead (http://www.mutantfactory.ouhsc.edu/protocols.asp). PCR primers used were as follows. The outer primer set: 5′-GTTGCTAGATGTCGGGTGGT-3′ (17689) and 5′-TTCTCAGTTGTCTTGGCACG-3′ (20685); the inner primer set: 5′-GAGTTAGGAATGCAGCCGAG-3′ (17736) and 5′-TGAACGCTGA-GAACGTGTTT-3′ (20606). The numbers given in the parentheses represent the 5′ end point of each primer on the cosmid B0414. We consequently obtained one *cpb-3* deletion mutant (*bt17*). To eliminate any additional mutations, backcrosses were performed at least five times. Sequence analysis indicated that the *bt17* allele carried a 1171-bp-long deletion, which corresponded to nucleotides 17845 through 19015 of the cosmid B0414.

Time course analysis of the germ cell number

To obtain a population of developmentally synchronized animals, L1 larvae that hatched from eggs during a 2-h interval were collected and transferred to a fresh plate. Animals were transferred to 25°C and allowed to develop for a defined period of time before fixation. Gonads were extruded and fixed with 3% formaldehyde/0.5× PBS/75% methanol at −20°C. After staining with DAPI, the number of germ cells was counted in the mitotic region, the transition zone, and the pachytene region. The mitotic/transition and the transition/pachytene boundaries were defined as circumferential rings of nuclei carrying ~80% crescent-shaped nuclei.

Staining of gonads with anti-CPB-3 antibodies

A fragment of *cpb-3* cDNA corresponding to amino acid residues 1 through 292 was cloned into the vector pET19b (Novagen) to create a 10xHis-CPB-3 fusion construct. The fusion protein was expressed in *Escherichia coli* BL21, purified using Ni-NTA agarose (Qiagen), and used as the antigen to raise rabbit polyclonal antibodies (Scrum). Anti-CPB-3 antibodies were purified by blot affinity purification. For histochemistry, dissected gonads were fixed and incubated with antibodies as described (Miller and Shakes, 1995). Affinity purified anti-CPB-3 was used at a 1:100 dilution and anti-FLAG M2 (Sigma) at a 1:2000 dilution. Images were captured by a Zeiss Axioplan 2 microscope equipped with a Hamamatsu cooled CCD camera and an attached FISH Imaging software and processed with Photoshop CS (Adobe). Western blot analysis was

performed essentially as described (Maruyama et al., 2005). Affinity purified anti-CPB-3 was used at a 1:500 dilution, anti-FLAG M2 antibody at a 1:20,000 dilution, and anti-actin antibody (Chemicon) at a 1:1000 dilution.

Immunoprecipitation

Immunoprecipitation of worm extracts was performed as described (Lee and Schedl, 2001). A total extract of a transgenic strain (*btIs2[daz-1-3xFLAG]; daz-1(tj3)*), in which the *daz-1(tj3)* mutation was rescued by integration of *daz-1* tagged with 3 copies of FLAG epitope at the C-terminus, was prepared by freezing and pressing in homogenizing buffer [20 mM HEPES (pH7.4), 250 mM NaCl, 2 mM EDTA, 5% glycerol, 0.5% Triton X-100, 1 mM DTT, 1 tablet/10 ml complete Mini (Roche)]. The extract was incubated with 1/100 volume of agarose beads coupled to specific antibodies for 1 h. Beads were washed three times with homogenizing buffer. Sample buffer for SDS-PAGE was added to the beads, and proteins were separated on 10% polyacrylamide gel, followed by detection using anti-CPB-3 and anti-FLAG M2 antibodies.

Results

The cpb-3 mutant shows multiple defects in germline development

To identify a new factor relevant to germline development, we performed a yeast two-hybrid screen using *C. elegans* DAZ-1 as bait. Among 282 positive colonies obtained, three contained cDNA clones that encoded the C-terminal region of CPB-3. We confirmed that these clones could activate the adenine reporter gene when co-expressed with the DAZ-1 bait clone but not with a control plasmid (Fig. 1A). CPB-3 is one of the four *C. elegans* CPEB homologs. Although circumstantial evidence has suggested that CPB-3 has a role in germline development (Luitjens et al., 2000), its function has not been clarified. We thus set out to analyze *cpb-3*.

To explore the function of *cpb-3* genetically, we isolated a *cpb-3* deletion mutant by the TMP/UV method (Yandell et al., 1994). The mutation, designated *bt17*, was an in-frame deletion that removed 1171 nucleotides from the *cpb-3* coding region. The deduced *bt17* allele encoded a truncated CPB-3 protein missing most of the conserved RRMs and the entire zinc finger domain (Fig. 1B; see below). Because these domains are essential for RNA binding in *Xenopus* CPEB (Hake et al., 1998), *cpb-3(bt17)* was likely to be a loss-of-function allele. Another *cpb-3* deletion allele, *tm1746*, was isolated and provided to us by the National Bioresource Project of Japan (http://shigen.lab.nig.ac.jp/c.elegans/index.jsp?lang=english). This allele lacked 568 nucleotides from the second exon (Fig. 1B). The *tm1746* mutation caused a frameshift to generate an aberrant ORF with a stop codon TGA at position 173. Because the *bt17* and the *tm1746* mutants displayed essentially the same phenotypes (see below), we mainly used the *bt17* allele in the following experiments, unless otherwise noted.

The *cpb-3* homozygote appeared normal in body structure and movement. It was fertile but had reduced brood size,

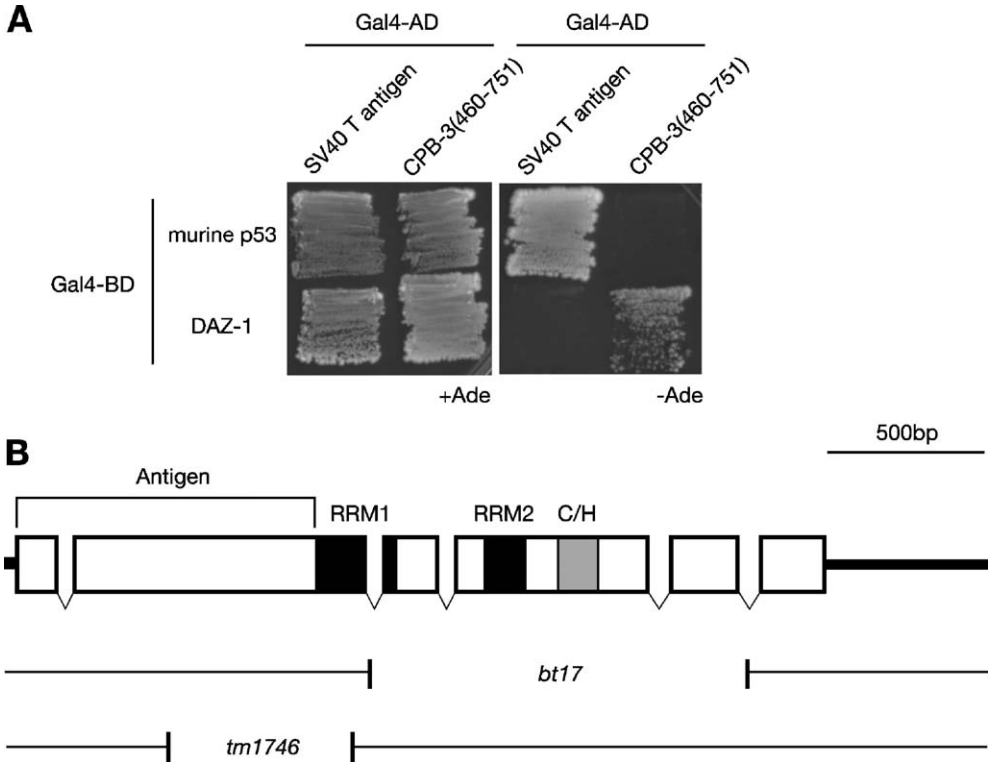

Fig. 1. Isolation of CPB-3 as a DAZ-1-interacting protein. (A) Interaction between CPB-3 and DAZ-1 in a yeast two-hybrid system. Yeast strain PJ69-4A carrying Gal4-BD-bait and Gal4-AD-prey plasmids were streaked on selective SD medium either supplemented with adenine or not. Growth in the absence of adenine indicates positive interaction of the bait and prey. Interaction of murine p53 and SV40 large T-antigen is shown as a positive control. (B) Genomic structure of the *cpb-3* gene. Exons are indicated by boxes, and the two RNA recognition motifs (RRMs) are blackened. The single shaded box represents the two consecutive cysteine/histidine zinc finger motifs comprising C4 and C2H2 residues. Regions deleted in the mutant alleles (*bt17* and *tm1746*) are illustrated under the gene structure. The N-terminal region of CPB-3 used as an antigen is indicated above the gene structure.

especially at high temperature. Whereas wild-type N2 laid on average about 280 eggs at 20°C and about 170 at 25°C, the *cpb-3* mutant laid about 170 eggs at 20°C and only about 30 at 25°C (Table 1). Furthermore, half of the eggs laid by *cpb-3* at 25°C showed embryonic lethality (Table 1). Because the defect was more prominent at 25°C, we refer to the characteristics of the *cpb-3* mutant observed at 25°C whenever we discuss its phenotypes hereafter. The *cpb-3* mutation was recessive and animals heterozygous for *cpb-3* (+/*bt17* or +/*tm1746*) showed normal brood size (data not shown). The *cpb-3(bt17)/cpb-3 (tm1746)* heterozygote showed similar phenotypes to each homozygote, confirming that deficiency of *cpb-3* was responsible for the mutant phenotypes.

To characterize possible germline defects in the *cpb-3* mutant, we precisely observed gonads of *cpb-3* hermaphrodites after DAPI staining. In wild-type adult gonads, germ cells display an orderly distal-to-proximal pattern reflecting their developmental stages. Germ cells proliferating mitotically are localized to the most distal region. As they migrate proximally, they initiate and execute meiosis. Next to the mitotic nuclei are crescent-shaped nuclei of the transition zone, which are preceded by thread-like pachytene nuclei and further by diakinesis nuclei (Fig. 2A, B). This pattern does not change in wild-type adults whether they are raised at 20°C or 25°C. In contrast, gonads of the *cpb-3* mutant showed multiple defects at 25°C, especially in old adults. Gonads of young *cpb-3* adults (48 h after hatching) were indistinguishable from wild-type gonads, except that they contained nuclei reminiscent of primary spermatocytes and had a slightly decreased number of diakinesis nuclei (4.3 ± 1.1 in wild type vs. 3.2 ± 1.6 in *cpb-3*) (Fig. 2C). However, gonads of old *cpb-3* adults (72 h after hatching) exhibited more prominent defects. They contained nuclei characteristic of the mitotic region, of the transition zone, and of the pachytene region, but these nuclei were packed much less densely compared to those in wild-type gonads. Moreover, pachytene nuclei occupied the proximal region of the old mutant gonad, replacing diakinesis nuclei (Fig. 2D). Apoptotic germ cells, stained by SYTO12, were increased in the old *cpb-3* mutant (3.1 ± 1.6 in wild type vs. 12.7 ± 8.8 in *cpb-3*), although this apparently could not explain the reduction of the number of germ cells in *cpb-3*. Germline apoptosis in the *cpb-3* mutant was observed only in the proximal region, and the *cpb-3* germ cells were already reduced in the distal pachytene region (data not shown).

Table 1
Progeny size of wild-type N2 and the *cpb-3* mutants

Genotype	20°C		25°C	
	Average brood size (%embryonic lethal)	*n*	Average brood size (%embryonic lethal)	*n*
N2	275.8 ± 43.8 (0.3)	9	165.5 ± 21.5 (0.9)	11
cpb-3(bt17)	174.1 ± 35.4 (6.0)	13	35.0 ± 13.1 (54.9)	20
cpb-3(tm1746)	158.4 ± 48.2 (10.9)	19	25.6 ± 14.3 (44.4)	17
cpb-3(bt17)/cpb-3 (tm1746)	189.9 ± 26.4 (3.5)	22	35.0 ± 15.0 (52.5)	22

Progeny size is shown as mean ± SD.

To obtain more chronological accounts of *cpb-3* phenotypes, we performed a time course analysis of the *cpb-3* germline. Oogenesis started at 44 h after hatching in both wild-type and *cpb-3* hermaphrodites, suggesting that germline development was not delayed in the *cpb-3* mutant up to this point. The sum of germ cells in the mitotic, transition and pachytene regions (designated as "total" in Fig. 2) continued to increase in the wild type until 68 h after hatching. The increase of germ cells in the *cpb-3* mutant was comparable to that in the wild type until 44 h after hatching, which corresponds to the young adult stage. Then, however, the number of germ cells in each stage stayed nearly constant or decreased slightly in *cpb-3* (Figs. 2E–H). The difference between wild type and *cpb-3* was most prominent at the pachytene stage in adult animals (52–76 h after hatching) (Fig. 2H). Progression from the pachytene to the diakinesis stage was suppressed at about 60 h after hatching in the *cpb-3* germ cells, and nearly one-third of the *cpb-3* population displayed no diakinesis nuclei at 72 h after hatching (data not shown). In addition, while spermatogenesis was completely switched to oogenesis at 48 h after hatching in the wild type, primary spermatocytes were still observed at 52 h after hatching in the *cpb-3* mutant (data not shown). These observations indicate that the *cpb-3* germline may develop normally up to the young adult stage but then all germ cells apparently arrest further development, except for a small portion of pachytene cells that can gradually mature into oocytes. This is, to our knowledge, a unique phenotype caused by a deficiency of a single gene, which may be regarded as a combination of three types of impairments: (1) inefficient proliferation and differentiation of germ cells in the mitotic region; (2) impaired switching from spermatogenesis to oogenesis or a compromised oocyte fate; and (3) a failure of pachytene cells in progressing into diakinesis.

CPB-3 is expressed in early meiotic regions of the hermaphrodite gonad

To examine the expression of CPB-3 protein, we raised polyclonal antibodies against the N-terminus of CPB-3 (Fig. 1B). The anti-CPB-3 antibodies reacted with a single polypeptide in a protein extract prepared from wild-type adult hermaphrodites, which appeared to have the molecular mass predicted for CPB-3 (84 kDa) (Fig. 3A). The *cpb-3(tm1746)* deletion mutant did not produce this polypeptide, supporting that it represented CPB-3 and that the antibodies were specific to it. The *cpb-3(bt17)* mutant expressed a possible truncated fragment of CPB-3 due to the in-frame deletion at a low level (Fig. 3A). CPB-3 was barely detected in an extract of *glp-4(bn2)* adult animals cultured at the restrictive temperature, which lacked germ cells, suggesting that CPB-3 was enriched in the germline at the adult stage.

To analyze a spatial distribution of CPB-3 in the germline, we immunostained dissected gonads with anti-CPB-3 antibodies. In gonads of either L4 or young adult wild-type hermaphrodites, CPB-3 was barely detectable at the most distal end, was gradually expressed in the mitotic region, and reached high levels in the transition zone and the pachytene region. Its

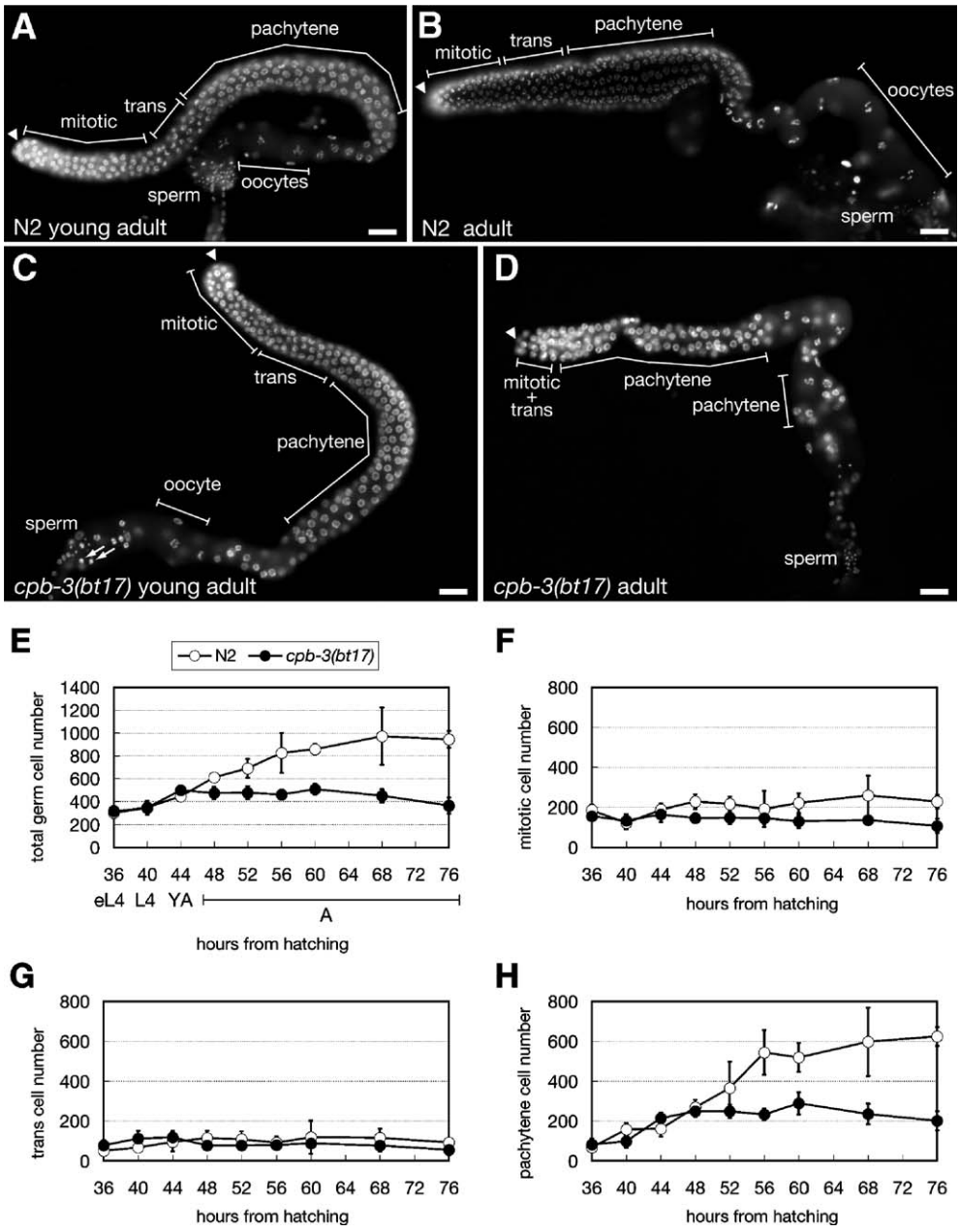

Fig. 2. Germline phenotypes of the *cpb-3* mutant. (A–D) DAPI-stained images of gonads extruded from hermaphrodites raised at 25°C. (A) Wild-type N2 young adult (48 h after hatching), (B) N2 adult (72 h after hatching), (C) *cpb-3(bt17)* young adult (48 h after hatching), and (D) *cpb-3(bt17)* adult (72 h after hatching). Arrowheads indicate the distal end of each gonad. Regions nesting either mitotic germ cells or each stage of meiotic cells are bracketed and annotated respectively. Arrows in panel C indicate primary spermatocytes. Scale bar: 20 μm. (E–H) Time course analysis of the number of germ cells in the gonad of N2 (open circle) or *cpb-3(bt17)* (closed circle) grown at 25°C. The numbers of total (i.e., F + G + H) germ cells (E), mitotic germ cells (F), germ cells in the transition zone (G), and pachytene germ cells (H) are shown. eL4 represents early L4 stage; YA, young adult stage; and A, adult stage. For wild-type N2, germline nuclei in 3–5 animals were counted. For *cpb-3* mutant, germline nuclei in 7–10 animals were counted. Error bars: standard deviation.

expression was then gradually decreased in the proximal pachytene region, and no staining was observed in mature sperm and oocytes (Figs. 3B, C). Staining of dissected gonads with anti-CPB-3 antibodies appeared specific because no substantial signal was detected in the *cpb-3(tm1746)* mutant (Fig. 3D). CPB-3 was stained predominantly in the cytoplasm, which is also the case with other CPEB homologs (Bally-Cuif et al., 1998; Chang et al., 1999; Luitjens et al., 2000; Thompson et al., 2005).

CPB-3 physically interacts with DAZ-1 in vivo

We examined relative localization of CPB-3 and DAZ-1 in a gonad. For this, we used a transgenic strain in which the sterility of *daz-1* was rescued by the transgene *btIs2*, which encodes DAZ-1 tagged with 3 copies of FLAG. This transgenic strain, detailed construction of which will be described elsewhere, was fertile and could produce oocytes (Fig. 4A, DAPI staining). DAZ-1-FLAG expressed from *btIs2* showed the same spatial

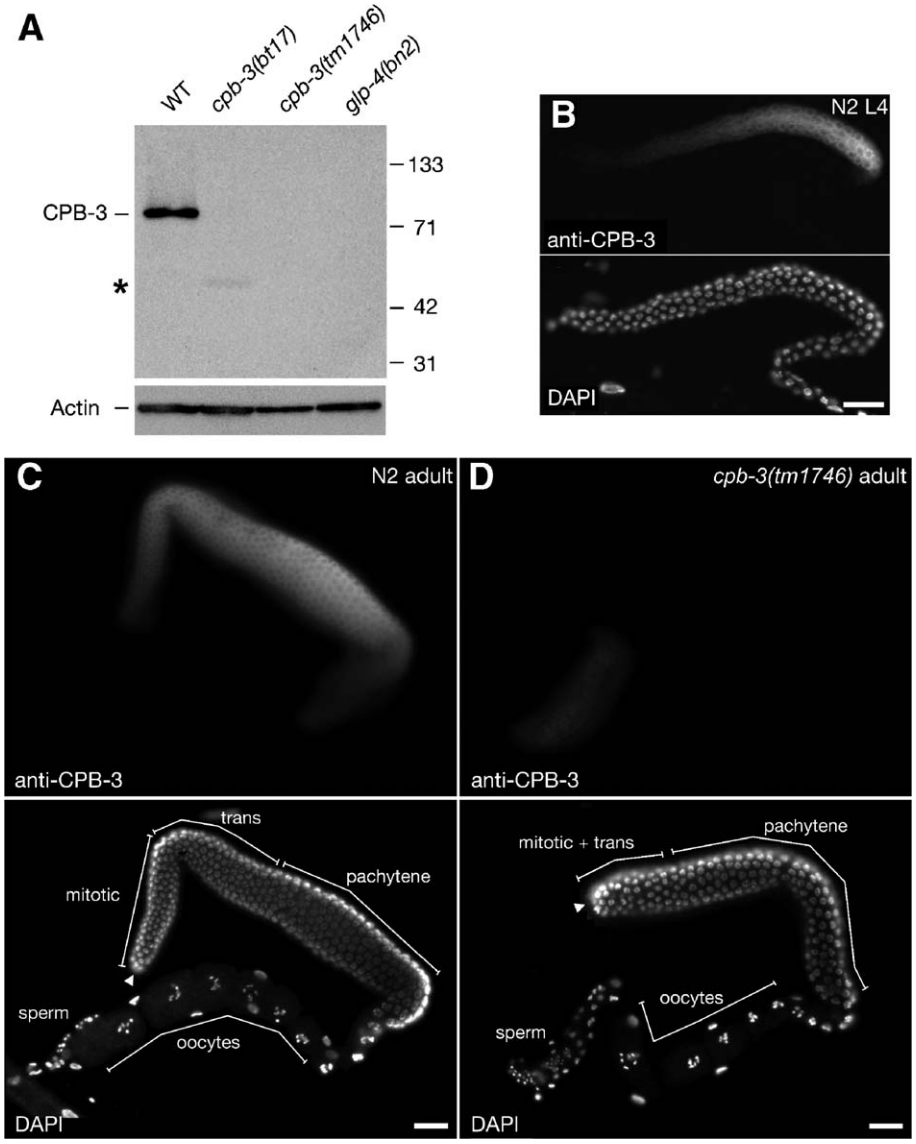

Fig. 3. Expression of CPB-3 protein in L4 larval and adult hermaphrodites. (A) Immunoblot detection of CPB-3 protein in extracts of N2, *cpb-3(bt17)*, *cpb-3(tm1746)*, and *glp-4(bn2)* animals. A total extract prepared from 120 adult hermaphrodites was loaded in each lane. The asterisk indicates possible truncated CPB-3(bt17) protein. Actin was measured as a loading control. (B–D) Fluorescent images of gonads stained with anti-CPB-3 antibodies and DAPI. (B) Wild-type N2 at the L4 larval stage, (C) N2 adult, and (D) *cpb-3(tm1746)* adult. Arrowheads indicate the distal ends of the gonads. Scale bar: 20 μm.

distribution as authentic DAZ-1 examined previously (Fig. 4A; Maruyama et al., 2005). By double staining of the *btIs2* strain with anti-CPB-3 and anti-FLAG antibodies, we found that the regions expressing DAZ-1 and CPB-3 partially overlapped each other (Fig. 4A). To corroborate the possible in vivo interaction between DAZ-1 and CPB-3, we performed immunoprecipitation assays using the same transgenic strain. We precipitated DAZ-1-3xFLAG from a worm extract using a monoclonal anti-FLAG antibody. Subsequent Western blot analysis with anti-CPB-3 antibodies revealed that CPB-3 was co-immunoprecipitated with DAZ-1, though in a small amount (Fig. 4B, left panels), but not at all with control IgG. Conversely, when we immunoprecipitated CPB-3 from the same worm extract using anti-CPB-3 antibodies, DAZ-1-3xFLAG was co-immunoprecipitated with CPB-3 (Fig. 4B, right panels). In each case, IgG beads precipitated a small amount of CPB-3 or DAZ-1, which was judged to be significantly less than that with anti-CPB-3 or anti-FLAG beads did. These results suggested that CPB-3 could interact with DAZ-1 in vivo.

Functional relation of cpb-3 with daz-1 and other genes involved in the sperm/oocyte switch or the mitosis/meiosis decision

To see whether CPB-3 acts in cooperation with or in opposition to DAZ-1, we constructed a *cpb-3; daz-1* double mutant. As shown previously, meiosis in the *daz-1* mutant was blocked at the pachytene stage during oogenesis (Karashima et al., 2000) (Fig. 5A). The *cpb-3; daz-1* hermaphrodite was sterile at any temperature (data not shown). In this mutant, the mitotic region, the transition zone, and the pachytene region were much smaller than those in the wild type and even smaller than the

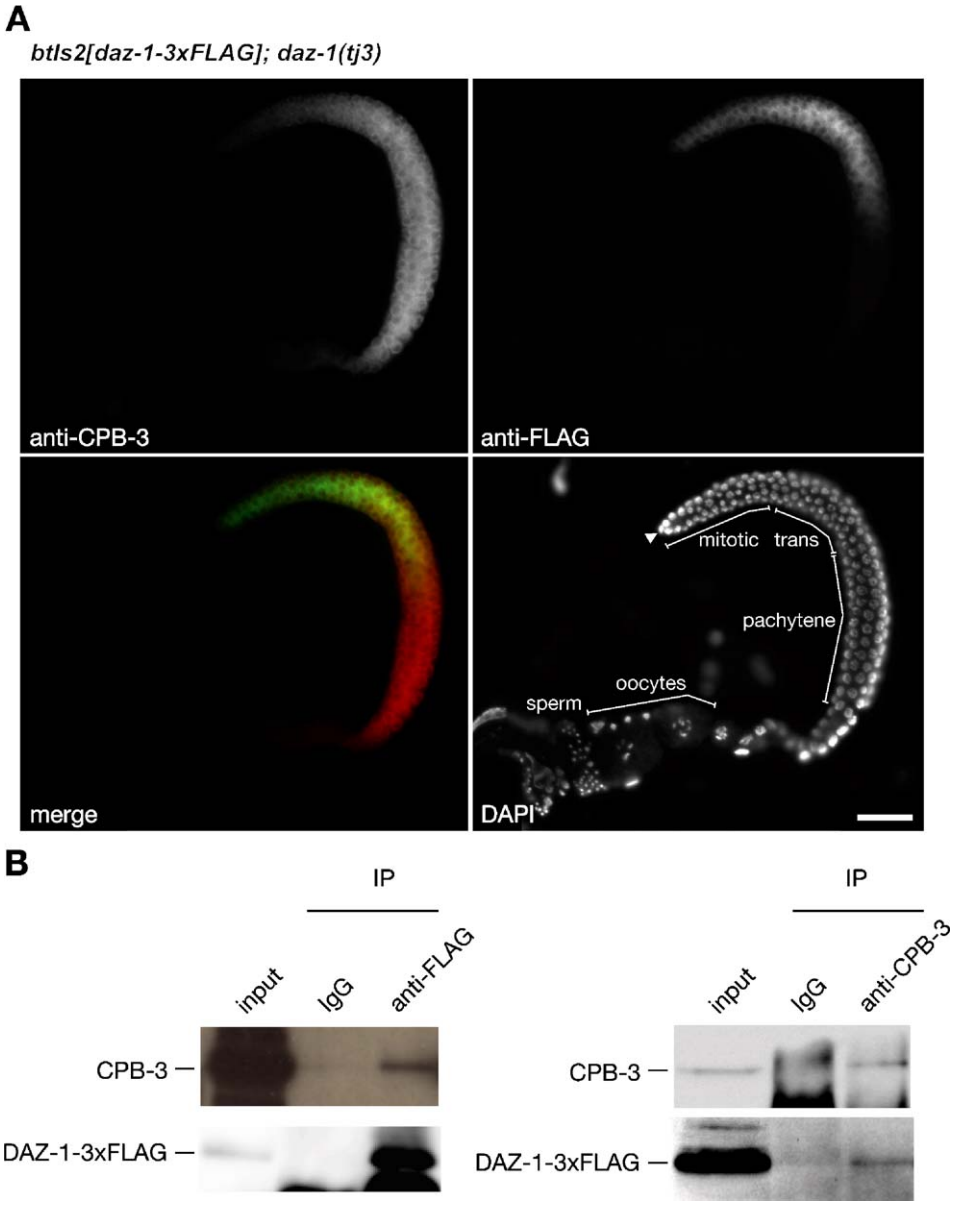

Fig. 4. Physical interaction of DAZ-1 and CPB-3 proteins. (A) Adult hermaphrodite gonad of *btIs2[daz-1-3xFLAG]; daz-1(tj3)* doubly stained for endogenous CPB-3 and transgenic DAZ-1-3xFLAG. Upper left panel, anti-CPB-3 staining; upper right panel, anti-FLAG staining; lower left panel, a merged image (CPB-3 in red and DAZ-1-3xFLAG in green); and lower right panel, DAPI staining. The arrowhead indicates the distal end of the gonad. Scale bar: 20 μm. (B) Immunoprecipitation with anti-FLAG antibody (left panels) and anti-CPB-3 antibodies (right panels). Loaded materials for the left panels (from left to right): 1.7% of the extract used for precipitation (input), IgG immunoprecipitate (IgG), and anti-FLAG immunoprecipitate. Loaded materials for the right panels (from left to right): 10% of the extract used for precipitation (input), IgG immunoprecipitate (IgG), and anti-CPB-3 immunoprecipitate. CPB-3 was detected with anti-CPB-3 antibodies and DAZ-1-3xFLAG with anti-FLAG antibody. Note that the exposure time varies among the panels.

cpb-3 mutant. In addition, the *cpb-3; daz-1* mutant germline was masculinized; i.e., nuclei undergoing spermatogenesis were seen even in the adult stage (Fig. 5B). These observations suggest that *cpb-3* and *daz-1* may share redundant roles in the sperm/oocyte switch and germline proliferation.

To genetically characterize the defect caused by the *cpb-3* mutation in the sperm/oocyte switch, we combined *cpb-3* with *fem-3*, which promotes spermatogenesis. In *fem-3(lf)* hermaphrodites, spermatogenesis does not occur, and only oocytes are produced (Hodgkin, 1986) (Fig. 5C). The *cpb-3(bt17); fem-3 (e2006)* germline showed defects similar to those in the *cpb-3* mutant except that it produced no sperm (Fig. 5D), indicating that *fem-3* is epistatic to *cpb-3*, and that *cpb-3* is important for oogenesis whether spermatogenesis takes place or not. The *cpb-3; daz-1; fem-3* triple mutant germline had oogenic pachytene nuclei but no sperm, suggesting that *cpb-3* functions upstream of *fem-3* to promote the sperm/oocyte switch (data not shown).

We next combined *cpb-3* and *fbf-1* mutations. FBF-1 and FBF-2 are nearly identical proteins that redundantly promote the sperm/oocyte switch by repressing *fem-3* (Zhang et al., 1997). The *fbf-1* mutant germline is slightly masculinized, i.e., about 1% of *fbf-1* hermaphrodites produce only sperm, while

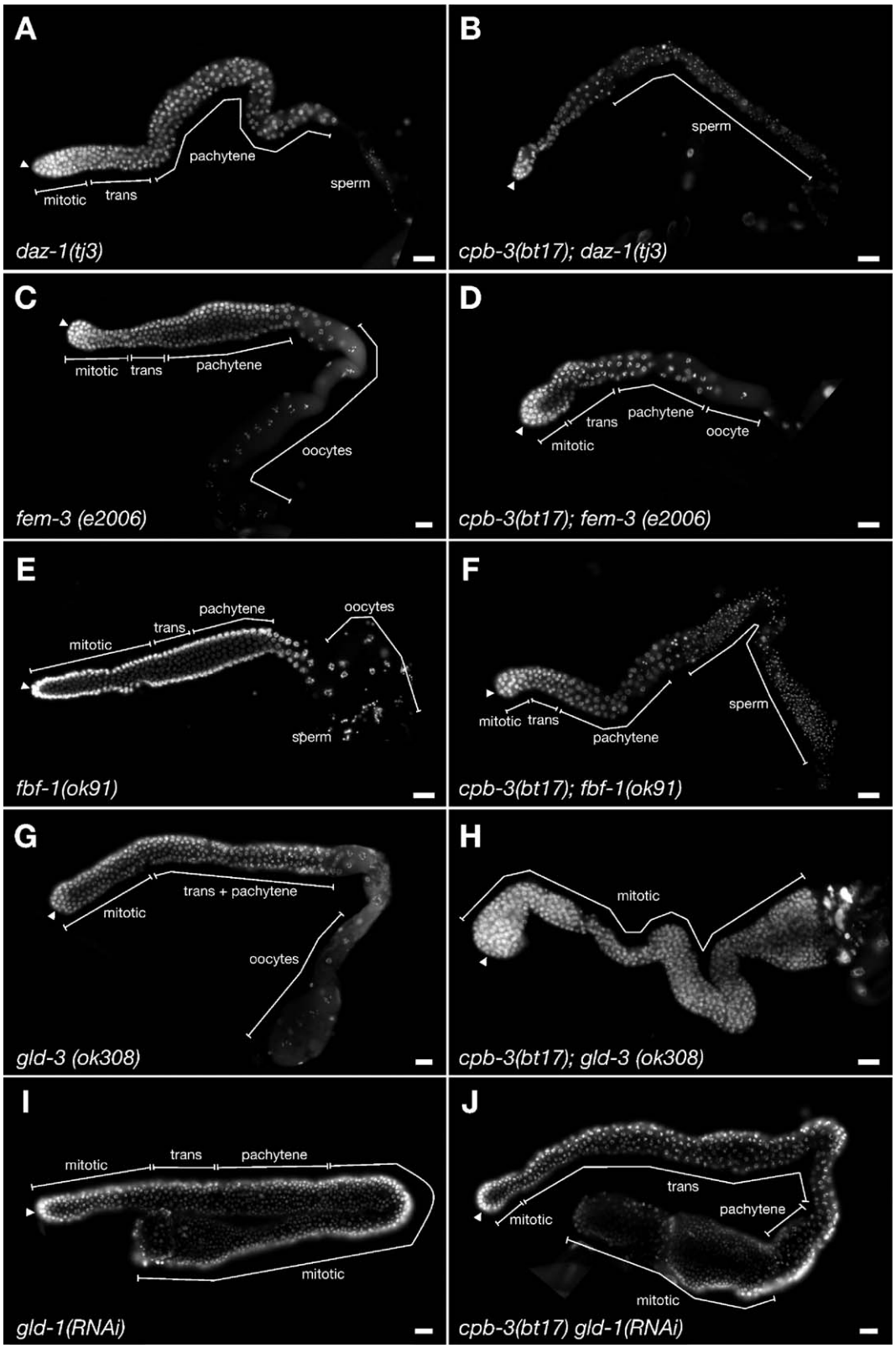

Fig. 5. Germline phenotypes of double mutants. DAPI-stained images of the gonads from adult hermaphrodites grown at 25°C for 72 h after hatching are shown. (A) *daz-1(tj3)*, (B) *cpb-3(bt17); daz-1(tj3)*, (C) *fem-3(e2006)*, (D) *cpb-3(bt17); fem-3(e2006)*, (E) *fbf-1(ok91)*, (F) *cpb-3(bt17); fbf-1(ok91)*, (G) *gld-3(ok308)*, (H) *cpb-3 (bt17); gld-3(ok308)*, (I) *gld-1(RNAi)*, and (J) *cpb-3(bt17) gld-1(RNAi).* Arrowheads indicate the distal end of each gonad. Scale bar: 20 μm.

99% of them produce both sperm and oocytes (Lamont et al., 2004) (Fig. 5E). The *cpb-3; fbf-1* double mutant was completely sterile at 25°C, and about half of *cpb-3; fbf-1* animals were sterile at 20°C (19/36). Sterile *cpb-3; fbf-1* animals showed masculinized germline: no oogenic nuclei were seen in their gonads, but instead, they displayed nuclei characteristic of

spermatogenesis (Fig. 5F). This apparent enhancement of the *fbf-1* masculinization phenotype by *cpb-3* suggests that *cpb-3* may have a positive role in the sperm/oocyte switch that is redundant with *fbf*. Unlike *fbf-1 fbf-2* mutant, however, *cpb-3; fbf-1* mutant did not develop a Glp (abnormal *g*erm*l*ine *p*roliferation) phenotype, suggesting that the role of *cpb-3* in germline is not strictly the same as *fbf*.

Finally, we examined genetic interactions of *cpb-3* with genes involved in the entry into meiosis. The GLD-1/NOS-3 and the GLD-2/GLD-3 pathways are known to promote entry to meiosis in a parallel manner (Eckmann et al., 2004; Hansen et al., 2004). For example, germ cells in the *nos-3 gld-3* double mutant repeat only mitosis without entering meiosis and eventually result in a germline tumor (Eckmann et al., 2004). We found that the *cpb-3; gld-3* double mutation caused ectopic germline proliferation (Fig. 5H). At 25°C, 23.3% (7/30) of *cpb-3; gld-3* mutant animals showed a tumorous germline with no sign of meiosis. In 40% (12/30) of them, germ cells entered meiosis in the distal region but resumed the mitotic cell cycle in the proximal region, generating a germ cell tumor. The rest (11/30) exhibited an apparent combination of the *gld-3* and *cpb-3* phenotypes: they generated no mature sperm like *gld-3* and exhibited pachytene arrest of oogenesis, though somewhat less intensively than *cpb-3* (data not shown). These results suggest that loss of *cpb-3* function may lower the activity of the GLD-1/NOS-3 pathway. Consistent with this idea, the *cpb-3; nos-3* mutant did not show such extensive germline proliferation (data not shown), and the *cpb-3* mutant subjected to *gld-1(RNAi)* showed a tumorigenic phenotype not stronger than that of *gld-1(RNAi)* animals, although the *cpb-3 gld-1(RNAi)* germ cells often passed a longer transition zone (Fig. 5J). However, enhanced ectopic germline proliferation such as seen in *cpb-3; gld-3* animals was not observed in *cpb-3 gld-2(RNAi)* either (data not shown). This may be explained by the proposition that *gld-2* and *gld-3* do not have identical roles in executing the mitosis/meiosis decision (Eckmann et al., 2004).

Discussion

Role of CPB-3 in germline development

This study has shown that a CPEB homolog, CPB-3, regulates oocyte production in *C. elegans*. Specifically, CPB-3 seems to promote the entry to and progression through meiosis after the young adult stage, as demonstrated by the time course analysis. We note that, in addition to the role in promoting progression of meiosis as proposed previously (Stebbins-Boaz et al., 1996; Tay and Richter, 2001), CPEB is likely to have a conserved function in the early steps of meiosis. It seems that *cpb-3* acts redundantly with *fbf-1* and possibly with *daz-1*, in the sperm/oocyte switch. It has been reported that human PUM2 (Pumilio homolog) and BOL (DAZ homolog) interact with each other (Moore et al., 2003). Genetic interaction between *cpb-3* and *fbf-1* shown in this study may suggest that functional interaction between DAZ- and PUF- family proteins is a conserved phenomenon in metazoans.

As mentioned in the Introduction, the other three CPEBs in *C. elegans* apparently function in spermatogenesis. RNAi for *cpb-1* blocks spermatogenesis at the primary spermatocyte stage, and the *fog-1* mutant makes functional oocytes but no sperm (Barton and Kimble, 1990; Luitjens et al., 2000). CPB-1 protein can be detected only in meiotic prophase cells undergoing spermatogenesis. The long isoform of FOG-1 protein, which is the functional isoform, is expressed solely in spermatogenic cells (Thompson et al., 2005), and *cpb-2* mRNA is virtually absent in oogenic cells (Luitjens et al., 2000). Because CPB-2, but neither CPB-1 nor FOG-1, appeared to interact with DAZ-1 in a two-hybrid assay like CPB-3 (our unpublished results), we examined phenotypes of the *cpb-3; cpb-2(RNAi)* animal. They were indistinguishable from those of the *cpb-3* mutant, and *cpb-2(RNAi)* alone gave no obvious phenotype, suggesting that *cpb-3* and *cpb-2* have no functional overlap (our unpublished results). Taken together, among four *C. elegans* CPEBs, only CPB-3 is likely to have an important function in oocyte production. This notion seems to be consistent with that CPEB homologs implicated in oogenesis in other species are more similar to CPB-3 than the remaining three (Luitjens et al., 2000; Mendez and Richter, 2001).

This and previous studies have implicated *Xenopus* CPEB, mouse mCPEB-1, *C. elegans* CPB-1, and *C. elegans* CPB-3 in the progression of meiosis. Inhibition of *Xenopus* CPEB blocks oocyte maturation (Stebbins-Boaz et al., 1996). In mCPEB-1 knockout mice, meiosis for both spermatogenesis and oogenesis arrests at the pachytene stage (Tay and Richter, 2001). *C. elegans* adult hermaphrodites deficient in *cpb-1* contain only spermatocytes that have failed to execute meiosis (Luitjens et al., 2000). As shown in this study, CPB-3 protein is highly expressed at the pachytene stage in the wild-type germline, and in adult *cpb-3* hermaphrodites, female meiosis is largely blocked. Altogether, the function of CPEB as a regulator of meiosis seems to be conserved among animal species. In addition, CPEB seems to play a role in germ cell differentiation. *Drosophila* CPEB Orb is required for proper localization and translation of *gurken* mRNA in dorsoventral axis formation and for translation of *oskar* mRNA in anteroposterior axis formation (Castagnetti and Ephrussi, 2003; Chang et al., 1999, 2001). In *C. elegans*, CPB-3 is implicated in the sperm/oocyte switch (this study), and FOG-1 is required for specification of the sperm fate (Barton and Kimble, 1990).

It is unclear why germline defects of the *cpb-3* mutant are severer at higher temperature. Because wild-type N2 exhibits somewhat reduced brood size at elevated temperature, gametogenesis may be unstable at higher temperature. This temperature effect and defects brought by the *cpb-3* mutation may act synergistically on germline development. Alternatively, CPB-3 may be included in a certain protein complex (discussed in the following section), which becomes thermo-labile if CPB-3 is missing.

Possible molecular function of CPB-3

In *Xenopus* oocytes, CPEB controls translation of c-*mos* mRNA, which encodes a MAP kinase kinase kinase.

Accumulation of c-Mos triggers activation of a MAP kinase cascade that culminates in MPF activation and meiotic maturation. In immature oocytes, CPEB acts as a translational repressor, forming a complex with Maskin and eIF4E (eukaryotic translation initiation factor 4E), which blocks assembly of a translation initiation complex. In contrast, CPEB acts as a translational activator during oocyte maturation. CPEB phosphorylated by Aurora kinase in response to the progesterone signal binds CPSF (cleavage and polyadenylation specificity factor), which recruits PAP to mRNA. This causes poly(A) elongation and translational activation of c-*mos* mRNA, together with dissociation of Maskin from eIF4E (Mendez and Richter, 2001). *Xenopus* CPEB has been reported to interact and cooperate with GLD-2 (Barnard et al., 2004), which is an atypical PAP first identified in *C. elegans* by a genetic screen (Kadyk and Kimble, 1998). To clarify the possible function of CPB-3 in poly(A) elongation, it will be informative to see which PAP may bind to CPB-3 in vivo or whether CPB-3 can recruit a PAP at all. *Drosophila* Orb is also thought to regulate poly(A) elongation of *oskar* mRNA positively in vivo (Castagnetti and Ephrussi, 2003). However, the phosphorylation sites by Aurora kinase are not conserved in invertebrate CPEB homologs. The *C. elegans* genome does not seem to encode a homolog of Maskin, whereas it encodes subunits of CPSF. Therefore, CPB-3 is unlikely to function as either a translational repressor or a dual regulator, although we cannot exclude the possibility that CPB-3 may repress translation of mRNA required for the entry to meiosis by a different molecular mechanism.

Xenopus CPEB is abundant in immature oocyte (prophase of meiosis I), but most of it is destroyed following oocyte maturation (after metaphase of meiosis I) (Mendez and Richter, 2001). Likewise, *C. elegans* CPB-3 protein is abundant in the pachytene region but almost undetectable at the diakinesis stage. *Xenopus* CPEB is phosphorylated by cdc2 kinase following oocyte maturation and brought to the proteasome pathway. Its degradation is dependent on the PEST box in the N-terminus (Mendez and Richter, 2001). The phosphorylation sites by cdc2 and the PEST box are conserved in CPB-3, implying the possibility that degradation of CPB-3 is controlled similarly to that of *Xenopus* CPEB.

Physical interaction of CPB-3 with DAZ-1

In this study, we have shown that CPB-3 can interact with DAZ-1 in vivo. However, the efficiency of co-immunoprecipitation between CPB-3 and DAZ-1 was not very high, and their expression patterns did not overlap entirely in the wild-type gonad. Therefore, only a small portion of CPB-3 and a small portion of DAZ-1 may form a complex when they are co-expressed in the transition zone through the middle pachytene region, which then regulates translation of some specific mRNAs. CPB-3 alone may also target other mRNAs in this area. In other regions, CPB-3 and DAZ-1 may act independently by binding to different target mRNAs. Identification of their target mRNAs will be critical to clarify these intriguing possibilities.

A recent study has shown that *Xenopus* DAZL indirectly promotes CPEB activity via RINGO/Spy and a phosphorylation site in CPEB (Padmanabhan and Richter, 2006). However, *C. elegans* has no apparent ortholog of RINGO/Spy, and the phosphorylation site is not conserved in CPB-3. Therefore, we suspect that *C. elegans* is unlikely to have a homologous regulatory pathway.

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